Express Mail No: EV 603556978 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial Number:	10/081,955
Filed:	February 20, 2002
Applicants:	George E. Seidel, Lisa Herickhoff, John Schenk
Title:	Multiple Sexed Embryo Production System for Mammals Using Low Numbers of Spermatozoa
TC/A.U:	1634
Examiner:	Carla J. Meyers
Assignee:	XY, Inc. and Colorado State University through its agent Colorado State University Research Foundation
Attorney Docket:	XY-Super-Cont2
Customer No.	33549

AFFIDAVIT UNDER 37 C.F.R. §1.132

UNITED STATES OF AMERICA	)
STATE OF COLORADO	) ss.
COUNTY OF LARIMER	)

I, George Seidel, a resident of LaPorte, in the County of Larimer, State of Colorado, duly sworn and under oath, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

I currently am employed as a University Distinguished Professor in the College of Veterinary Medicine, Department of Biomedical Sciences, at Colorado State University in Fort Collins, CO, USA. I have been employed in this capacity continuously since 1971, and currently am tenured. My duties in this position include conducting research in the field of animal reproduction. A specific research focus of mine is the area of artificial insemination of mammals, including aspects involving superovulation. My duties require me to be extensively familiar with practices and developments in this area. Accordingly, I have at various times reviewed such practices and developments. Such review has included the review of relevant academic and scholarly literature, my own empirical experimentation, and extensive discussion and collaboration with other researchers in this area. I also have authored or co-authored several publications relevant to this area, including: Seidel, G.E. Jr., 1981, "Superovulation and Embryo Transfer in Cattle", Science 211:351-358; Funston, R.N., Seidel, G.E. Jr., 1995, "GnRH Increases Cleavage

Rates of Bovine Oocytes Fertilized In Vitro", Biol. Reprod., 53:539-543; and Seidel, G.E. Jr., "Use of Sexed Bovine Sperm for In Vitro Fertilization and Superovulation", Animal Reproduction and Biotechnology Lab, CSU, Proceedings of the 2000 CETA/ACTE Convention, Charlottetown, Prince Edward Island, August 2000, pp. 22-24.

As a result of my experience and expertise in this area, I have knowledge and skill that is at least representative of those of ordinary skill in the art pertaining to the artificial insemination of mammals, including aspects involving superovulation.

Based on the foregoing, I am able to make the following statements.

Prior to the present invention, the prevailing view among those having at least ordinary skill in the art in the area of artificial insemination of mammals has been that sperm transport is compromised in superovulated mammals. Such compromised sperm transport may represent an impediment to achieving successful fertilization using artificial insemination techniques. As a result, where other factors are held equal, it has been demonstrated that artificial insemination may tend to result in lower rates of successful fertilization for superovulated mammals than for non-superovulated mammals. This view is supported by many empirical observations reported in many academic and professional publications over many years of practice. As examples of the general acceptance of this view, two representative publications are discussed as follows.

Hawk, H.W., 1988, "Gamete Transport in the Superovulated Cow", Theriogenology 29:125-142. A copy of this publication is attached to this affidavit as Exhibit "A". The Abstract of this publication expressly states in the first sentence that reduced efficiency of sperm transport in superovulated cows can be inferred from their consistently lower fertilization rates as compared to non-superovulated cows, and the Abstract later states that among other factors, insemination with high numbers of sperm has been required to increase the fertilization rate above 90%. In addition, the Introduction describes prior studies by Dowling and Umbaugh showing impaired gamete transport in superovulated cattle. Under the heading Sperm Transport in Superovulated Cattle, many points are made supporting the concept of compromised sperm transport in cattle, including the consistently lower fertilization rates observed in superovulated cows as compared to non-superovulated cows, the tendency of indicators such as the lack of accessory sperm to confirm such compromised sperm transport, and several additional studies further confirming observation of this phenomenon.

Saacke, R.G., DeJarnette, J.M., Bame, J.H., Karabinus, D.S., and Whitman, S.S., 1998, "Can Spermatozoa With Abnormal Heads Gain Access to the Ovum in Artificially Inseminated Super- and Single-Ovulating Cattle?", Theriogenology 50:117-128. A copy of this publication is attached to this affidavit as Exhibit "B". On page 121, it is shown on Table 1 that while superovulated cows indeed produced more ova than single ovulated cows, nevertheless superovulated cows exhibited a nearly 20% lower fertilization rate than single ovulated cows. Moreover, the percentage of fertilized ova with accessory sperm cells – an indicator of adequate sperm transport – was more than 50% lower in superovulated cows than single ovulated cows. On page 124, it is

speculated that superovulation may affect the hardness of the zona pellucida of the ovum, potentially readily impacting the pregnancy rate by altering the selectivity of this barrier and of the sperm transport system in general. It is further noted that the possibility of insemination with very high numbers of sperm may be required to optimize sperm accessibility to the ovum in superovulated cattle.

As a result of the prevailing view regarding compromised sperm transport in superovulated mammals, those having at least ordinary skill in the art prior to the present invention would not have been able to combine known techniques for insemination with low numbers of sperm with known techniques for superovulation and have a reasonable expectation of success of achieving fertilization rates of at least 35%, at least 41%, at least 50%, or at least 90% of a typical unsorted insemination dosage. Moreover, to the extent that the prevailing trend prior to the present invention has been to use high or very high numbers of sperm for the artificial insemination of superovulated mammals, the prior art may be considered to teach away from the practice of utilizing low numbers of sperm with known techniques for superovulation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or any patent issued thereon.

DATED this 12th day of December, 2005.

George Seidel

UNITED STATES OF AMERICA)
STATE OF COLORADO)ss.
COUNTY OF LARIMER)

SUBSCRIBED AND SWORN to before me in the County of Larimer, State of Colorado, United States of America, by George Seidel this 12th day of December, 2005. WITNESS my hand and official seal pursuant to the authority vested in me as a Notary Public by the State of Colorado.

Notary Public

My Commission Expires: 2.19.06

BARBARA L. HOBACK NOTARY PUBLIC STATE OF COLORADO

GAMETE TRANSPORT IN THE SUPEROVULATED COW

H. W. Hawk

U.S. Department of Agriculture, Agricultural Research Service,
Reproduction Laboratory, Animal Science Institute,
Beltsville Agricultural Research Center,
Beltsville, MD 20705

ABSTRACT

Reduced efficiency of sperm transport in superovulated cows can be inferred from fertilization rates, which generally average about 65%, or about 20% lower than those found in single-ovulating cattle. Also, most fertilized ova recovered from superovulated cattle have no accessory sperm in the zona pellucida, which suggests minimal numbers of sperm at the site of fertilization. Low fertility of either cows or bulls reduces the fertilization rate, probably through an effect on survival of sperm or efficiency of sperm transport in the cow. Other factors that affect fertilization rate, presumably also acting through sperm survival or transport, include age of the cow, season of the year, interval after calving, stage of the estrous cycle at which the superovulation treatment is begun, total dose of FSH used, number and timing of artificial inseminations, and site of deposition of the inseminate. Use of purified FSH to induce superovulation has improved the fertilization rate markedly in some instances, and insemination with high numbers of sperm in fresh undiluted semen has raised the fertilization rate above 90%. The apparent inhibition of sperm transport in superovulated cows may be mediated through changed patterns of steroid hormone secretion.

Ova seem to pass through the oviducts faster in superovulated cows than in single-ovulating cows. The rate of passage may be shortened by a day or more in some cases, but the proportion of superovulated cows with hastened ovum transport is not known, and the consequences of rapid transport on survival of embryos has not been ascertained.

INTRODUCTION

Gamete transport in superovulated cattle has been a concern to investigators and practitioners of embryo transfer since the early days of superovulation. In 1949, Dowling (1) recovered only 38% of potential ova from superovulated cows, and only 38% of the recovered ova were fertilized. He attributed the low fertilization rate to an absence of sperm at the site of fertilization and the low ovum recovery rate to accelerated passage of ova through the oviducts. In the same year, Umbaugh (2) obtained fertilization rates of 50-55% in superovulated cows; he believed that some feature of his hormone treatment limited fertilization, and he noted that ova from superovulated cows sometimes descended prematurely into the uterus.

Acknowledgment: The author thanks Linda Neuenhahn for typing the manuscript.

SPERM TRANSPORT

Measures of sperm transport include the fertilization rate, numbers of accessory sperm in the zona pellucida, fertilization rate after ligation of one or both oviducts at a specific location and time after insemination, and enumeration of sperm in the various segments of the reproductive tract at one or more time intervals after insemination.

Fertilization indicates success of sperm transport and fertilization failure generally indicates failure of some phase of sperm survival or transport. Number of accessory sperm will generally be positively correlated with fertilization rate. Ligation of the oviducts, with subsequent determination of the fertilization rate, has provided information on the time required for fertilizing sperm to have passed the point of ligation. Numbers of sperm in the various segments of the reproductive tract have provided information on the speed of transport and on the distribution and fate of sperm in the female.

Each of these measures of sperm transport has advantages and disadvantages, but the total information obtained has provided a reasonably good picture of sperm transport in single-ovulating cattle (3). Sperm transport in superovulated cattle is another matter. In 1976, Baker and Polge (4), in a review of fertilization in cattle, stated that little quantitative information was available on sperm transport in superovulated cows. Unfortunately, direct measurement of sperm numbers in the reproductive tract of superovulated cows is still almost totally lacking. A few significant points are given below regarding sperm transport in single-ovulating cows.

Sperm Transport in Single-Ovulating Cattle

Dobrowolski and Hafez (5) deposited 2 billion sperm in the external cervical os of heifers, necropsied the heifers at 1, 8 or 20 hours after insemination, flushed each segment of the reproductive tract and counted sperm in the flushings. Within each of the three time intervals, most of the sperm recovered from the tract were in the vagina, with decreasing numbers of sperm being found in the cervix, uterus and oviducts. Further, the total number of sperm remaining in the reproductive tract declined steadily over time. At 1 hour after insemination, only 13% of the sperm were recovered from the entire tract, at 8 hours, 4%, and at 24 hours, less than 1%.

Results of several experiments on distribution of sperm in the reproductive tract of cows have revealed a retrograde movement of sperm, even after deposition of semen in the uterus by artificial insemination (6, 7, 8). Mitchell et al. (7) and Nelson et al. (9), by collecting all mucus and urine discharged from cows after insemination, determined that most sperm were lost by drainage to the exterior in cervical mucus. The major part of this loss occurred between 4 and 8 hours after insemination (7).

Sperm have been found in the oviducts of cattle within a few minutes after insemination (3), but such sperm are probably not involved in fertilization of ova. In rabbits, sperm that are transported to the oviducts within a few minutes after insemination are disrupted and mostly dead (10). In cattle, Hunter and Wilmut (11, 12) ligated and transected the oviducts of heifers at or near the uterotubal junction at various time intervals between 6

and 31 hours after mating. Results of these studies established that about 8 hours are required for sperm capable of fertilizing ova to reach the isthmus of the oviducts, that sperm are held in the caudal 2 cm of the isthmus until ovulation, and that some of the sperm then move to the site of fertilization near the isthmic-ampullary junction. Any physiological usefulness of the sperm transported to the oviducts of cattle within a few minutes after insemination is not known.

Sperm Transport in Superovulated Cattle

Great variation among females has always been encountered, especially in cattle and sheep, in the number of sperm in any particular segment of the reproductive tract at a given time after insemination (13). In superovulated cattle, variation in number of sperm reaching the oviducts probably accounts for the commonly seen distribution of fertilization rates and number of accessory sperm: numerous accessory sperm in ova from a few cows, with all ova being fertilized, very few accessory sperm in ova from the majority of cows, with many or most ova being fertilized, and no accessory sperm in ova from some cows, with few or no ova being fertilized.

Apparently, only one study has been reported in which sperm were recovered from the oviducts of superovulated cows (14). Eight cows were each inseminated with 13 million motile sperm, and one oviduct was removed 30 h after onset of estrus, presumably about 18 h after insemination. The remaining oviduct was later flushed for recovery of ova. An average of 99 sperm was found in the isthmus of the eight cows; an average of 23 sperm was found in the ampullae of four of the cows, with no sperm being found in ampullae of the other four cows. Cleaved ova were recovered from each of the four cows in which sperm had been found in the ampulla, but ova were cleaved in only one of four cows in which sperm had not been found in the ampulla.

Other investigators have studied sperm transport in gonadotropin-treated calves. Howe and Black (15) found sperm in oviducts of several gonadotropin-treated calves less than 1 h after insemination. At 16-21 h, each of three control calves and three of four gonadotropin-treated calves had sperm in the oviducts. Lineweaver et al. (16) found sperm in the oviducts of gonadotropin-treated calves 4 h after deposition of sperm in the uterus by laparotomy but not after deposition of semen at the external cervical os.

Because of the lack of direct study of sperm transport in superovulated cows, the efficiency of sperm transport in these animals relative to that in single-ovulating cattle must be inferred from fertilization rates and accessory sperm numbers.

The fertilization rate is almost always higher in single-ovulating cows than in superovulated ones. In single-ovulating cows inseminated artificially, the fertilization rate is generally 85% to 90% (17, 18), with an occasional figure approaching 100% (19). By comparison, the fertilization rates for superovulated cows in which several thousand ova have been examined in commercial embryo transfer units have averaged 65% to 71% (20, 21, 22). In a study in which a direct comparison was made of fertilization rates in unsuperovulated and superovulated cows, Seidel et al. (23) reported fertilization rates of 89% in unsuperovulated cows and 70% in superovulated

cows ($P<0.05$), even though the superovulated cows were inseminated three times with six times as many sperm as were the unsuperovulated cows.

A number of factors, such as unfertilizable eggs or failure of sperm capacitation, could account for the high rate of fertilization failure in superovulated cows. However, the available evidence points to a lack of sperm at the site of fertilization as the most frequent cause of fertilization failure. The evidence includes the following: unfertilized ova seldom have sperm attached to the zona pellucida (23); only a small proportion of fertilized ova in artificially inseminated cows have accessory sperm in the zona pellucida at the time of ovum recovery (19, 24); the fertilization rate can approach 100% when high numbers of sperm are used for insemination (25).

Accessory sperm as well as fertilization rate should provide an indication of efficiency of sperm transport. The number of accessory sperm in cleaved ova from unsuperovulated artificially inseminated cows ranges from none to several hundred, but the number is generally in the range of 5 to 75 (19, 26). In unsuperovulated single-ovulating cows, accessory sperm were present about 3 days after estrus in 54 of 55 cleaved ova (98%) from first-service cows and in 23 of 30 cleaved ova (77%) from repeat-breeder cows (19). In the same study, accessory sperm were found in only 49 of 269 cleaved ova (18%) from 21 superovulated first-service cows and in only 6 of 55 cleaved ova (11%) from repeat-breeder cows. In a study with superovulated heifers, 95 total ova, cleaved or uncleaved, were collected from 13 animals inseminated either 12 or 24 h after the beginning of estrus; only 15 (16%) of the ova had accessory sperm (24).

Other evidence of a probable detrimental effect of superovulation on sperm transport comes from a negative relationship in FSH-treated cows between ovulation rate and accessory sperm numbers. In 1969, Bellows et al. (27) treated groups of beef heifers with total FSH doses of 3.12, 6.25, 12.5 and 25 mg; numbers of corpora lutea averaged 1.1, 2.1, 8.0, and 14.6, respectively. For the groups with cows averaging 1.1 or 2.1 corpora lutea, the combined fertilization rate was 93%; for the groups with cows averaging 8.0 or 14.6 corpora lutea, the fertilization rate was 82%. Accessory sperm numbers indicated that as the number of ovulations increased, the number of accessory sperm decreased. It might be questioned whether the cows with 1.1 or 2.1 corpora lutea were actually superovulated, but the results demonstrated a depressing effect of superovulation on fertilization rate and accessory sperm numbers. In superovulated beef heifers that averaged about 21 ovulations per animal, Hafez et al. (28) found no definite relationship between number of ovulations and fertilization rate.

Caution must be exercised in using fertilization rates and accessory sperm numbers as an indication that superovulation greatly suppresses sperm transport. The number of sperm at the site of fertilization is not known for either superovulated or unsuperovulated cows, and complete fertilization failure is found in approximately 15% of both types of cows (17, 18, 20). In one study that included both superovulated and unsuperovulated cows (19), an average of 26 total sperm was associated with ova of superovulated cows; most of these sperm had fertilized an ovum and were represented by an embryo, with very few accessory sperm being seen. An average of 40 total sperm was associated with the ovum of each unsuperovulated cow; all but the single fertilizing sperm were seen as accessory sperm. It is not known whether the

multiple ova in superovulated cows were contacted by a higher proportion of available sperm than was the single ovum in unsuperovulated cows.

Despite the unknown factors involved in comparing the efficiency of sperm transport between superovulated and unsuperovulated cows, it seems likely that sperm transport is moderately inhibited in superovulated cows. Sperm transport is known to be inhibited in superovulated sheep. Evans and Armstrong (29) flushed a uterine horn and oviduct of ewes 24 h after insemination and recovered fewer sperm from superovulated ewes than from unsuperovulated ewes. In another study, superovulation, compared with natural ovulation, reduced the number of sperm in the oviducts, uterus and anterior segments of the cervix at 3 and 23 h after mating (Table 1). Especially significant was the reduction in sperm numbers in the anterior third of the cervix at 3 h after mating; sperm populations must be established by 2 or 3 h in the anterior cervix of ewes to assure a high fertilization rate (13). Low sperm numbers in the anterior cervix at 3 h were followed by low sperm numbers in the oviducts at 23 h, near the time of ovulation (Table 1). Low sperm numbers in superovulated ewes were associated with high proportions of sperm in the cervix and uterus that were immotile, dead or had disrupted membranes. In other ewes of the same study, superovulation reduced the fertilization rate; 47 superovulated ewes had 575 ova, of which 274 (48%) were cleaved whereas 46 unsuperovulated ewes had 62 ova, of which 44 (71%) were cleaved.

Table 1. Effect of type of ovulation and time after mating on number of sperm in the reproductive tract of the ewe[a]

Time after mating and type of ovulation[b]	Number of sperm recovered				
			Cervix[c]		
	Oviducts	Uterus	Ant.	Mid.	Post.
		$(10^3)^d$	(10^6)	(10^6)	(10^6)
3 h					
Natural ovulation	105	75	1.2	8.9	24.1
Superovulation	61	23	0.4	5.0	27.3
23 h					
Natural ovulation	504	30	1.4	1.9	0.8
Superovulation	62	6	0.1	0.2	0.3

[a] Adapted from reference 30.

[b] Eight ewes per treatment group.

[c] Anterior, middle and posterior thirds of the cervix.

[d] Multiplier for means for the uterus and cervix.

Although superovulation reduced the efficiency of sperm transport in ewes, the reduction was apparently not as severe as that caused by some other treatments, such as regulation of estrus with prostaglandin $F_{2\alpha}$ or progestogen (13).

The problem with sperm transport in superovulated ewes appeared to be transport through the cervix (Table 1), perhaps caused by disruption of sperm membranes. Whether similar problems of sperm survival and transport might occur after intrauterine insemination of superovulated cows is not known.

Factors Affecting Fertilization Rates in Superovulated Cows

Several factors influence the fertilization rate in superovulated cattle. Because efficiency of sperm transport to the site of fertilization is almost certainly a major factor in determining the fertilization rate, these factors will be discussed briefly.

Fertility of Cows. At least two direct comparisons have been made of fertilization rates in superovulated fertile and infertile (repeat-breeder) cattle. Elsden et al. (31) obtained a fertilization rate of 72% in 18 fertile cows but only 35% in 23 infertile cows. Similarly, Greve (32) obtained fertilization rates of 82% in 13 fertile cows and 41% in 11 repeat-breeders.

Hasler et al. (20) compared fertilization rates in 666 "healthy" cows and 318 "infertile" cows. The rates were 66% and 42%, respectively ($P<0.05$); 14% of healthy cows and 51% of infertile cows had no fertilized ova. These differences suggest that superovulated infertile cows as a group have more problems with sperm transport or survival than do superovulated healthy cows. This is not surprising because unsuperovulated repeat-breeder cows often have lower fertilization rates than do unsuperovulated first-service cows (3, 31).

Fertility of Sires. Callaghan and King (33) superovulated beef heifers with PMSG, timed estrus with $PGF_{2\alpha}$, and inseminated the heifers with fresh semen from bulls that were consistently above or below average for non-return rate. Fertilization rates, ascertained by examination of ova recovered at necropsy 3 or 4 days after insemination, averaged 89% for 355 ova from heifers inseminated with semen from bulls of high fertility and 70% for 155 ova from heifers inseminated with semen from bulls of lower fertility.

Newcomb (34) compared fertilization rates in superovulated cows inseminated with frozen semen from a bull of above average fertility and a bull of below average fertility. Fertilization rates of ova collected non-surgically 7 days after estrus were 83% and 58%, respectively. These differences are similar to those between high and low fertility bulls after insemination of unsuperovulated heifers (3).

Age of Cow. Hasler et al. (20), working with data from 880 cows aged 3 years or older, reported a decline in fertilization rate of healthy cows after about 10 years of age and of infertile cows after about 6 years of age. Lerner et al. (22), with data from 872 cows, found that fertilization rate decreased linearly with increasing age of donor.

Season. In Pennsylvania, fertilization rates of healthy cows were significantly higher from May through October than November through April. For infertile cows, the highest fertilization rates were obtained in August to October and the lowest in November to January (20).

Interval after Calving. Hasler et al. (20) obtained higher fertilization rates from cows 151 to 300 days after calving (71%) than from cows less than 150 days (65%) or more than 300 days (66%).

Stage of Estrous Cycle. The day of the estrous cycle on which FSH treatment was started has affected the fertilization rate to some extent. Hasler et al. (20) found significantly higher fertilization rates when FSH treatment began on day 8, 9, 10 or 13 of the cycle (average of 69%) than when treatment began on day 11 or 12 (average of 62%). Lindsell et al. (35) reported a higher fertilization rate when FSH treatment started on day 9 (78%) than on day 6 or day 12 (57%). These percentages were calculated from total and fertilized ova listed by the authors (35).

Number of Previous Superovulations. Hasler et al. (20) reported data on 35 cows that were superovulated five times or more. Fertilization rates were highest, averaging 77%, for the first three superovulations, then declined to an average of 64% for the fourth and fifth superovulations and to 46% for the fifth through tenth superovulations. However, the fertilization rates after the fifth superovulation may have been distorted by some cows with higher fertilization rates being dropped from embryo collection.

Prostaglandin and FSH Treatments. Administration of 50 mg of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) in three divided doses 6 h apart resulted in cleavage of 62% of ova compared with 51% when 50 mg of $PGF_2\alpha$ was administered in two divided doses (36).

Donaldson (37) treated 222 cows with dinoprost tromethamine and 223 cows with cloprostenol to regress the corpus luteum in FSH-treated cows. The proportion of ova that cleaved was 75.5% after dinoprost and 67.4% after cloprostenol ($P=0.019$). However, the higher proportion of cleaved ova recovered from dinoprost-treated cows was accompanied by a higher proportion of degenerate embryos, so that the number of transferable embryos after dinoprost or cloprostenol was nearly identical.

Lerner et al. (22) reported that fertilization rate increased linearly with increasing dose of FSH. The rate of increase was 1.7% per mg of total dose of FSH, which ranged from 30 to 44 mg. The beneficial effect of increasing dose of FSH compensated partially for the adverse effects of advancing age of cows. A different result was reported by Elsden and Kessler (38) with Nelore Zebu cattle; total FSH dosages of 24, 36 and 50 mg resulted in fertilization rates of 60%, 44% and 29%, respectively.

Time and Number of Inseminations and Number of Sperm. Critser et al. (39) inseminated a total of 76 superovulated heifers with one ampule of semen at 12 h after detection of estrus, three ampules at 12 h or one ampule at each of 0, 12 and 24 h. The overall fertilization rate was 59%, and there were no significant differences due to number of ampules of semen used.

West et al. (40) and Donaldson (41) analyzed the effect of insemination regimen on fertilization of many thousands of ova from several hundred cows. Number of inseminations varied from one to three, and number of units of frozen semen used ranged from one to four. Fertilization rates varied from 58% to 77%. West et al. (40) recommended inseminating twice with one straw of semen at each breeding, and Donaldson (41) concluded that one insemination with one unit of semen 12 h after onset of estrus was satisfactory.

Schiewe et al. (42) inseminated cows of four groups with two units of frozen semen at 12, 24, 36 or 48 h after onset of estrus. Cows of a fifth group were inseminated at each time interval. Eggs were collected about 7 days after estrus. Fertilization rates were as high for cows inseminated at 12 or 24 h as for those inseminated at all intervals.

In the studies cited above on number and time of inseminations, the total number of sperm deposited in each female within 24 h after the beginning of estrus was always less than 100 million. In an attempt to increase the number of embryos obtained per cow, an experiment was done in this laboratory (25) in which 12 cows were inseminated with freshly collected semen (4.4 billion total sperm per cow). The cows yielded 197 ova, of which 183 (93%) were fertilized. From 56 control cows inseminated with one to three units of commercially available frozen semen (averaging 70 million total sperm per cow), 947 ova were collected, of which 502 were fertilized (53%, $P<0.01$). The number of motile sperm deposited in experimental cows was at least 100-fold higher than the number in the control cows. Part of the increase in fertilization rate could have been caused by factors other than sperm number because control cows were inseminated with frozen-thawed semen deposited only in the uterine body whereas experimental cows were inseminated with fresh semen that was deposited several centimeters into the uterine horns as well as in the uterine body. However, the principal reason for the high fertilization rate in experimental cows was probably the high number of sperm used.

Site of Semen Deposition. In single-ovulating cattle, the site of semen deposition, within the bounds of the anterior cervix, uterine body or posterior part of the uterine horns, has generally had little effect on fertility (43). However, Moller et al. (44), using inseminates of only 2.0 or 2.5 million total sperm, found non-return rates to be about 5% higher after deposition of semen in the uterine body than after deposition in the anterior third of the cervix. In a recent study with single-ovulating cows, semen was deposited in the uterine body or deep in one uterine horn, 15-20 cm anterior to the uterine body, either on the side of impending ovulation or on the opposite side (19). No differences were found among sites of semen deposition in fertilization rate or number of accessory sperm. In another study, sperm deposited deep in one uterine horn were distributed throughout the reproductive tract within 2 hours (45). After insemination of single-ovulating cattle, it appears that sperm capable of fertilizing ova are moved to both oviducts regardless of the site of semen deposition in the uterus.

In superovulated cows, the deposition of semen in one uterine horn has resulted in decreased fertilization rates in the opposite oviduct of some cows. In one experiment (19), 28 cows, 21 first-service and 7 repeat-breeders, were inseminated by deposition of semen 15-20 cm into one uterine horn. Over all 28 cows, fertilization rates were 74% in the oviduct on the side of insemination and 58% in the opposite oviduct ($P<.005$, Table 2).

However, the difference in fertilization rate was due almost entirely to four cows in which the fertilization rate on the side of insemination was 93% and on the opposite side, 19%. Apparently, sperm were moved with only low efficiency from one uterine horn to the opposite oviduct in 4 of 28 cows (14%). In these four cows, sperm were presumably moved out of the uterine horn of insemination into the uterine body, cervix and vagina, but were not transported anteriorly through the opposite uterine horn.

Results of the other experiment (46) in which cows were inseminated in one uterine horn were similar to the results just discussed (Table 2). Data were not given on the proportion of cows that contributed to the difference in fertilization rate between inseminated and non-inseminated sides of the reproductive tract.

Number of Follicles Ovulating. Shea et al. (47), recovering embryos and counting corpora lutea at surgery, noted a decline in fertilization rate from 84% in 164 beef cattle with 1-10 ovulations to 78% in 193 cattle with 11-20 ovulations and 71% in 100 cattle with 21 or more ovulations. Working with slaughtered beef heifers, Hafez et al. (28) noted low fertilization rates in animals that ovulated few ova as often as in those that ovulated many ova, and Callaghan and King (33) classified animals by number of corpora lutea, ranging from less than 5 to more than 25, and found no significant variation in fertilization rate among groups. However, these investigators worked with fewer cattle than did Shea et al. (47).

Table 2. Fertilization rates in superovulated cows inseminated in one uterine horn

Reference	Cattle (no.)	Uterine horn[a]	Ova (no.)	Cleaved ova (no.)	Cleaved ova (%)
19	28[b]	AI	243	180	74
		Non-AI	247	144	58
46	9	AI	68	59	87
		Non-AI	47	33	70

[a] Semen was deposited 15-20 cm into the AI horn in reference 19 and 5 cm into the AI horn in reference 46.

[b] Includes 21 first-service cattle and 7 repeat-breeders.

Time of Ovulation. Apparently, ovulation begins, in most cattle treated to superovulate, about 24 h after the beginning of estrus (48, 49, 50). In those studies, follicles were observed at necropsy, surgery or laparoscopy. Most large follicles had ovulated by 12 h after initiation of ovulation (50). Wise et al. (51) reported a somewhat wider range in ovulation time. Based on ovariectomy at 24, 48 or 72 h after injection of $PGF_2\alpha$, a majority of animals had begun to ovulate before estrus was detected; 7 of 10 had begun to ovulate at 24 h after $PGF_2\alpha$, 8 of 10 at 48 h and 9 of 10 at 72 h. Behavioral estrus was seen only at 72 h, which is later than most FSH-treated cattle come into estrus after $PGF_2\alpha$ administration. Earlier, Hafez et al. (28), by daily palpation of the ovaries of superovulated cows, had concluded that ovulations sometimes occurred over a period of several days.

Ova originating from the early ovulations observed by Wise et al. (51) might be too old to be fertilized by the time sperm reached the site of fertilization. Also, early ovulations might be part of the reason for generally elevated progesterone levels at estrus in superovulated cows, as discussed below.

Hormone Secretion. As would be expected from the growth and development of numerous follicles and corpora lutea in superovulated cows, ovarian hormone secretion in these animals differs from that in single-ovulating cattle. The height and duration of the LH surge are similar in superovulated and in single-ovulating cattle (52), but blood plasma estrogen levels are much higher in superovulated cattle before and around the time of estrus (53, 54) and plasma progesterone concentrations are higher during estrus (54, 55). As the luteal phase begins, progesterone secretion rises quickly to high levels, and the amount secreted is related to the number of ovulations (54).

Greve et al. (56) examined the blood progesterone and LH profiles in superovulated dairy cattle around the time of estrus and classified the profiles as normal or deviating from normal. Cows with normal profiles had high fertilization rates, whereas those with profiles that deviated from normal for one or both hormones had much lower ovulation rates and fertilization rates.

Donaldson (52) also reported that normal timing of the LH surge was required for production of high numbers of transferrable embryos, presumably reflecting in part a high fertilization rate. Donaldson (52) suggested that an endogenous FSH surge nearly coincident with the LH surge increased the number of embryos recovered and that a properly timed LH surge increased the number of transferrable embryos.

The LH activity that is inherent in commercial FSH and PMSG preparations appears to reduce the fertilization rate in superovulated cows. Donaldson et al. (55) and Donaldson and Ward (57) superovulated cattle with an FSH preparation from which they had removed most of the LH by column chromatography. In one experiment, the purified FSH increased the ovulation rate from 52 to 74% (Table 3) and more than doubled the number of transferrable embryos per donor cow. Donaldson et al. (55) added LH to their purified FSH preparation and reduced the fertilization rate. Earlier, Murphy et al. (58) added LH to a commercial FSH preparation and reduced the ovulation rate and fertilization rate.

Donaldson and Ward (59), after obtaining fertilization rates of 48% in cattle superovulated with commercial FSH and 80% and 84% in cattle superovulated with two purified FSH preparations, concluded that low fertilization rates in superovulated cattle result from effects of LH. Progesterone concentrations in blood were significantly higher during estrus in cows treated with commercial FSH than in cows treated with purified FSH (55). The LH in commercial FSH preparations may have stimulated progesterone secretion during estrus, and the progesterone may have interfered with some phase of sperm survival or transport in superovulated cows. Barrios et al. (60) reduced the fertilization rate in superovulated cows by administering progesterone during estrus.

Table 3. Fertilization rates in cattle superovulated with commercial or purified porcine FSH[a]

FSH preparation	Ova and embryos recovered per cow (no.)	Embryos recovered per cow (no.)	Fertilization rate (%)[b]
Commercial	11.1	5.8	52
Purified	12.1	9.0	74

[a] Results from 130 cows. Adapted from reference 55.

[b] Calculated from numbers of embryos and ova per cow.

Additional data on ovum fertilization after superovulation of cattle with purified FSH is given in Table 4. The 151 cattle were predominantly Holsteins, representing a cross-section regarding reproductive health as described by Hasler et al. (20). They were superovulated with a purified FSH preparation described by Armstrong and Opavsky (61), inseminated with three units of frozen semen by professional A.I. technicians, and flushed for embryo recovery 7 days after estrus by personnel of Em Tran, Inc., Elizabethtown, PA. The fertilization rate of 65% (Table 4) is 4 percentage units higher than the 61% reported by Hasler et al. (20) for combined healthy and infertile cattle used by Em Tran and superovulated with commercial porcine FSH.

OVUM TRANSPORT

Dowling (1) superovulated cattle and necropsied them at various time intervals after insemination. In cattle superovulated with PMSG and necropsied 2 to 4 days after insemination, only about one-third of potential ova were recovered. The low recovery rate was ascribed to accelerated passage of ova through the oviducts, although no data were presented to support that assumption. According to Hamilton and Laing (62), embryos of single-ovulating cows enter the uterus about 96 h after ovulation.

Table 4. Fertilization rate in cattle superovulated with purified FSH [a]

No. cows	No. embryos and uncleaved ova	No. embryos	Fertilization rate (%)
151	1343	873	65

[a] Folltropin, Vetrepharm Inc., London, Ontario. Data supplied by J. F. Hasler, Em Tran, Inc., Elizabethtown, PA.

Hafez et al. (28), working with superovulated Hereford heifers, reported the percentage of ova in the oviducts or uterus at various days after the time of ovulation, as estimated by rectal palpation. At 2 days, all ova of three heifers were in the oviducts. At 3, 4, 5 and 6 days after ovulation, 39%, 83%, 93% and 92% of the ova from a total of 29 heifers had reached the uterus. These data indicated that most ova entered the uterus on the third day or early on the fourth day after ovulation, which was probably slightly earlier than would be expected in unsuperovulated animals. Later, El Banna and Hafez (63) made a direct comparison of ovum transport in superovulated and unsuperovulated Hereford heifers. At various time intervals from less than 10 h to more than 72 h after ovulation, as determined by rectal palpation of the ovaries, ova in oviducts of superovulated heifers seemed to have moved slightly farther toward the uterus than had ova in unsuperovulated heifers. By about 72 h, ova of three superovulated heifers had entered the uterus, whereas those of three unsuperovulated heifers had not.

McKenzie and Kenney (64) recovered 78 one-cell ova from the uterus of heifers; 28 of the ova subsequently cleaved in vitro, suggesting that ova had been transported through the oviducts before the first cleavage.

Data of Moore (65) and Newcomb et al. (66) on ovum transport in superovulated cattle are given in Table 5. The day of estrus was day 0. If it is assumed that animals were in heat about 6 h, on the average, before estrus was detected and that the middle of the time span of ovulation was about 30 h after the beginning of estrus, then the time of ovulation is day 1. The time from ovulation to recovery of ova would be 24 h shorter than each day listed in the table; i.e., day 2 would be about 24 h after the middle of the time span of ovulation.

Data of both Moore (65) and Newcomb et al. (66) show that most ova were still in the oviducts on day 3, about 48 h after ovulation (Table 5). The data in Table 5 indicate that more than half of the ova entered the uterus between day 3 and day 4, or between 48 and 72 h after ovulation. These times are similar to those of Hafez et al. (28) and El Banna and Hafez (63).

Table 5. Proportion of ova recovered from superovulated cows that were in the oviducts on various days after estrus[a]

Day after estrus[b]	Moore (65)			Newcomb et al. (66)		
		Ova recovered			Ova recovered	
	Cattle (no.)	Eggs No.	% in uterus	Cattle (no.)	Eggs No.	% in uterus
2	22	61	0	---	---	---
2.5	16	62	0	---	---	---
3	16	52	15	13	146	4
3.5	9	42	24	---	---	---
4	13	46	65	---	---	---
4.5	7	34	71	---	---	---
5	---	---	---	33	354	83
6	---	---	---	29	281	86
7	---	--	--	33	307	92
8	---	---	---	15	163	93

[a] Remainder of the ova were recovered from the oviducts.

[b] Estrus = Day 0.

Between day 4 and day 7, small numbers of ova continued to enter the uterus (Table 5), but 7% and 8% of the ova recovered on days 7 and 8 were still in the oviducts. Betteridge et al. (67) examined 65 superovulated cows for location of ova and embryos on days 10-16 and found that 6.1% of the total ova and embryos recovered were still in the oviducts. Apparently, a few percent of ova remain indefinitely in the oviducts and would not be recovered by non-surgical uterine flushes of live animals. It is also possible that ova remain indefinitely in the oviducts of a small proportion of unsuperovulated cattle.

Newcomb et al. (66) found no difference in rate of transport of fertilized and unfertilized eggs into the uterus.

When embryos and ova enter the uterus, they are not immediately scattered through the length of the uterine horns, but the majority remain near the uterotubal junction for a few days. Newcomb et al. (66) ligated the uterine horns 10 cm from the uterotubal junction on days 6, 7 and 8 after estrus and found that 73% of ova and embryos were located in this segment of the horns; 20% were elsewhere in the uterus and 7% were still in the oviducts.

It is difficult to assess the frequency or significance of rapid transport of ova through the oviducts of superovulated cattle. Rapid transport of ova has been mentioned sufficiently often for one to conclude that it occurs, but the proportion of cows in which it occurs and the average number of hours that transport is hastened cannot be calculated with

certainty. One problem is that the assumed time of entry of ova into the uterus of unsuperovulated cows is based on few animals. As indicated by Newcomb et al. (66), it would seem desirable to study ovum transport and the time of entry of ova into the uterus in both superovulated and unsuperovulated cattle.

Whether rapid transport of ova through the oviducts is detrimental to the future development of embryos is not clear. However, deliberately hastening the entry of ova into the uterus by flushing them out of the oviducts and into the uterine horns at day 3 (estrus = day 0) apparently caused degeneration of embryos (66).

The probable increase in rate of movement of eggs through the oviducts of at least some superovulated cattle may be caused by the higher blood progesterone concentration at estrus in superovulated than in unsuperovulated cattle (55) and in the rapid rise in progesterone secretion in most superovulated cattle (53, 54, 68). It is known that exogenous progesterone hastens egg transport through the oviduct in both naturally ovulating and superovulating cattle (60, 69, 70).

REFERENCES

1. Dowling, D. F. Problems of the transplantation of fertilized ova. J. Agric. Sci. 39:374-396 (1949).
2. Umbaugh, R. E. Superovulation and ovum transfer in cattle. Am. J. Vet. Res. 10:295-305 (1949).
3. Hawk, H. W. Transport and fate of spermatozoa after insemination of cattle. J. Dairy Sci. 70:1487-1503 (1987).
4. Baker, R. D. and Polge, C. Fertilization in swine and cattle. Can. J. Anim. Sci. 56:105-119 (1976).
5. Dobrowolski, W. and Hafez, E.S.E. Transport and distribution of spermatozoa in the reproductive tract of the cow. J. Anim. Sci. 31:940-943 (1970).
6. Suga, T. and Higaki, S. Studies on uterine secretions in the cow. II. Distribution of spermatozoa and seminal plasma after intra-uterine inseminations in the reproductive tract of the cow during oestrus. Bull. Natl. Inst. Anim. Ind. (Japan) 24:41-48 (1971).
7. Mitchell, J. R., Senger, P. L. and Rosenberger, J. L. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. J. Anim. Sci. 61:956-967 (1985).
8. Larsson, B. and Larsson, K. Distribution of spermatozoa in the genital tract of artificially inseminated heifers. Acta Vet. Scand. 26:385-395 (1985).
9. Nelson, V. E., Aalseth, E. P., Hawman, C. H., Adams, G. D., Dawson, L. J. and McNew, R. W. Sperm discharge and distribution within the cow's reproductive tract after A.I. J. Anim. Sci. 65:401 (1987) Abstr.
10. Overstreet, J. W. Transport of gametes in the reproductive tract of the female mammal. In Hartmann, J. F., (ed) Mechanism and Control of Animal Fertilization. Academic Press, New York, 1983, pp. 499-543.

11. Hunter, R.H.F. and Wilmut, I. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Develop. 24:597-608 (1984).

12. Wilmut, I. and Hunter, R.H.F. Sperm transport into the oviducts of heifers mated early in estrus. Reprod. Nutr. Develop. 24:461-468 (1984).

13. Hawk, H. W. Sperm survival and transport in the female reproductive tract. J. Dairy Sci. 66:2645-2660 (1983).

14. Wiley, H. E., Bowen, M. J., Massey, J. M., Amoss, M. S., Blake, R. W. and Kraemer, D. C. The effect of FSH on sperm transport in cattle. Theriogenology 17:113 (1982) Abstr.

15. Howe, G. R. and Black, D. L. Spermatozoan transport and leucocytic responses in the reproductive tract of calves. J. Reprod. Fertil. 6:305-311 (1963).

16. Lineweaver, J. A., Hafez, E.S.E., Ehlers, M. H., Dickson, W. M. and King, J. R. Sperm transport in cattle and gonadotropin-treated calves. Cornell Vet. 60:372-382 (1970).

17. Diskin, M. G. and Sreenan, J. M. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J. Reprod. Fertil. 59:463-468 (1980).

18. Roche, J. F., Boland, M. P. and McGeady, T. A. and Ireland, J. J. Reproductive wastage following artificial insemination of heifers. Vet. Record 109:401-404 (1981).

19. Hawk, H. W. and Tanabe, T. Y. Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. J. Anim. Sci. 63:551-560 (1986).

20. Hasler, J. F., McCauley, A. D., Schermerhorn, E. C. and Foote, R. H. Superovulatory responses of Holstein cows. Theriogenology 19:83-99 (1983).

21. Donaldson, L. E. Effect of insemination regimen on embryo production in superovulated cows. Vet. Record 117:35-37 (1985).

22. Lerner, S. P., Thayne, W. V., Baker, R. D., Henschen, T., Meredith, S., Inskeep, E. K., Dailey, R. A., Lewis, P. E. and Butcher, R. L. Age, dose of FSH and other factors affecting superovulation in Holstein cows. J. Anim. Sci. 63:176-183 (1986).

23. Seidel, G. E., Jr., Elsden, R. P., Nelson, L. D. and Hasler, J. F. Methods of ovum recovery and factors affecting fertilization of superovulated bovine ova. In Sreenan, J. R., (ed) Control of Reproduction in the Cow. M. Nijhoff, The Hague, 1978, pp. 268-280.

24. Diop, P.E.H., Bousquet, D. and King, W. A. Artificial insemination and fertilization in superovulated heifers. Theriogenology 27:223 (1987) Abstr.

25. Hawk, H. W., Conley, H. H., Wall, R. J. and Whitaker, R. O. Attempts to improve fertilization rates in superovulating cows. J. Anim. Sci. 65:400 (1987) Abstr.

26. Segerson, E. C. and Libby, D. W. Ova fertilization and sperm number per fertilized ovum for selenium and vitamin E-treated Charolais cattle. Theriogenology 17:333-341 (1982).

27. Bellows, R. A., Anderson, D. C. and Short, R. E. Dose-response relationships in synchronized beef heifers treated with follicle stimulating hormone. J. Anim. Sci. 28:638-644 (1969).

28. Hafez, E.S.E., Sugie, T. and Gordon, I. Superovulation and related phenomena in the beef cow. I. Superovulatory responses following PMS and HCG injections. J. Reprod. Fertil. 5:359-379 (1963).

29. Evans, G. and Armstrong, D. T. Reduction of sperm transport in ewes by superovulation treatments. J. Reprod. Fertil. 70:47-53 (1984).

30. Hawk, H. W., Cooper, B. S. and Conley, H. H. Inhibition of sperm transport and fertilization in superovulating ewes. Theriogenology 28:139-153 (1987).

31. Elsden, R. P., Nelson, L. D. and Seidel, G. E., Jr. Embryo transfer in fertile and infertile cows. Theriogenology 11:17-25 (1979).

32. Greve, T. Embryo transplantation in cattle. Non-surgical recovery of embryos from repeat breeders. Acta Vet. Scand. 21:26-33 (1980).

33. Callaghan, B. D. and King, G. J. Determination of the fertilization rate of A.I. sires. Theriogenology 14:403-410 (1980).

34. Newcomb, R. Investigation of factors affecting superovulation and non-surgical embryo recovery from lactating British Friesian cows. Vet. Record 106:48-52 (1980).

35. Lindsell, C. E., Murphy, B. D. and Mapletoft, R. J. Superovulatory and endocrine responses in heifers treated with FSH-P at different stages of the estrous cycle. Theriogenology 26:209-219 (1986).

36. Donaldson, L. E. The effect of prostaglandin F_2 alpha treatments on superovulated cattle on estrus response and embryo production. Theriogenology 20:279-285 (1983).

37. Donaldson, L. E. Comparison of Cloprostenol and Dinoprost Tromethamine for the control of estrus in bovine embryo transfer. Theriogenology 21:1019-1022 (1984).

38. Elsden, R. P. and Kessler, R. M. Superovulation of Nelore cattle and heifers. Theriogenology 19:127 (1983) Abstr.

39. Critser, J. K., Rowe, R. F., Del Campo, M. R. and Ginther, O. J. Embryo transfer in cattle: factors affecting superovulatory response, number of transferable embryos, and length of post-treatment estrous cycles. Theriogenology 13:397-406 (1980).

40. West, G., West, C., Risley, D. and Donaldson, L. Effect of breeding regime on percent ova fertilized in superovulated cows. Theriogenology 21:273 (1984) Abstr.

41. Donaldson, L. E. Effect of insemination regimen on embryo production in superovulated cows. Vet. Record 117:35-37 (1985).

42. Schiewe, M. C., Looney, C. R., Johnson, C. A., Hill, K. G. and Godke, R. A. Transferable embryo recovery rates following different insemination schedules in superovulated beef cattle. Theriogenology 28:395-406 (1987).

43. Olds, D. Insemination of the cow. In Salisbury, G. W., VanDemark, N. L. and Lodge, J. R., (ed) Physiology of Reproduction and Artificial Insemination of Cattle. W. H. Freeman, San Francisco, 1978, pp. 555-576.

44. Moller, K., Macmillan, K. L. and Shannon, P. Site of insemination and subsequent non-return rates in cows. N.Z. J. Agric. Res. 15:252-254 (1972).

45. Larsson, B. Transuterine transport of spermatozoa after artificial insemination in heifers. Anim. Reprod. Sci. 12:115-122 (1986).

46. Pallares, A., Zavos, P. M. and Hemken, R. W. Fertilization rates and embryonic development in superovulated cattle inseminated in different sites within the reproductive tract. Theriogenology 26:709-719 (1986).

47. Shea, B. F., Hines, D. J., Lightfoot, D. E., Ollis, G. W. and Olson, S. M. The transfer of bovine embryos. In Rowson, L.E.A., (ed) Egg Transfer in Cattle. Commission European Communities, Luxembourg, 1976, pp. 145-152.

48. Maxwell, D. P., Massey, J. M. and Kraemer, D. C. Timing of ovulations in the superovulated bovine. Theriogenology 9:97 (1978) Abstr.

49. Shea, B. F. Recovery of bovine follicular oocytes and their fertilization in a recipient animal. Theriogenology 9:101 (1978) Abstr.

50. Yadav, M. C., Walton, J. S. and Leslie, K. E. Timing of the onset and duration of ovulation in superovulated beef heifers. Theriogenology 26:509-521 (1986).

51. Wise, T., Vernon, M. and Maurer, R. R. Endocrine and ionic changes in preovulatory bovine follicles. J. Anim. Sci. 61 (Suppl. 1):368 (1985) Abstr.

52. Donaldson, L. E. LH and FSH profiles at superovulation and embryo production in the cow. Theriogenology 23:441-447 (1985).

53. Booth, W. D., Newcomb, R., Strange, H., Rowson, L.E.A. and Sacher, H. B. Plasma oestrogen and progesterone in relation to superovulation and egg recovery in the cow. Vet. Record 97:366-369 (1975).

54. Saumande, J. Concentrations of luteinizing hormone, oestradiol-17β and progesterone in the plasma of heifers treated to induce superovulation. J. Endocrinol. 84:425-437 (1980).

55. Donaldson, L. E., Ward, D. N. and Glenn, S. D. Use of porcine follicle stimulating hormone after chromatographic purification in superovulation of cattle. Theriogenology 25:747-757 (1986).

56. Greve, T., Callesen, H. and Hyttel, P. Endocrine profiles and egg quality in the superovulated cow. Nord. Vet.-Med. 35:408-421 (1983).

57. Donaldson, L. E. and Ward, D. N. Effects of luteinizing hormone on embryo production in superovulated cows. Vet. Record 119:625-626 (1986).

58. Murphy, B. D., Mapletoft, R. J., Manns, J. and Humphrey, W. D. Variability in gonadotrophin preparations as a factor in the superovulatory response. Theriogenology 21:117-125 (1984).

59. Donaldson, L. E. and Ward, D. N. LH effects on superovulation and fertilization rates. Theriogenology 27:225 (1987) Abstr.

60. Barrios, D. R., McConnell, S., Bowen, M. J. and Kraemer, D. C. Effects of progesterone (P_4) and estrogen (E_2) on fertilization failure and early embryonic mortality in superovulated cows. Theriogenology 23:176 (1985) Abstr.

61. Armstrong, D. T. and Opavsky, M. A. Biological characterization of a pituitary FSH preparation with reduced LH activity. Theriogenology 25:135 (1986) Abstr.

62. Hamilton, W. J. and Laing, J. A. Development of the egg of the cow up to the stage of blastocyst formation. J. Anat. 80:194-204 (1946).

63. El-Banna, A. A. and Hafez, E.S.E. Egg transport in beef cattle. J. Anim. Sci. 30:430-432 (1970).

64. McKenzie, B. E. and Kenney, R. M. In vitro culture of bovine embryos. Am. J. Vet. Res. 34:1271-1275 (1974).

65. Moore, N. W. The control of time of oestrus and ovulation and the induction of superovulation in cattle. Australian J. Agric. Res. 26:295-304 (1975).

66. Newcomb, R., Rowson, L.E.A. and Trounson, A. O. The entry of superovulated eggs into the uterus. In Rowson, L.E.A., (ed) Egg Transfer in Cattle. Commission European Communities, Luxembourg.

67. Betteridge, K. J., Eaglesome, M. D., Randall, G.C.B. and Mitchell, D. Collection, description and transfer of embryos from cattle 10-16 days after oestrus. J. Reprod. Fertil. 59:205-216 (1980).

68. Saumande, J., Tamboura, D. and Chupin, D. Changes in milk and plasma concentrations of progesterone in sows after treatment to induce superovulation and their relationships with number of ovulations and of embryos collected. Theriogenology 23:719-731 (1985).

69. Rowson, L. E. Methods of inducing multiple ovulation in cattle. J. Endocrinol. 7:260-270 (1951).

70. Crisman, R. O., McDonald, L. E. and Thompson, F. N. Effects of progesterone or estradiol on uterine tubal transport of ova in the cow. Theriogenology 13:141-154 (1980).

CAN SPERMATOZOA WITH ABNORMAL HEADS GAIN ACCESS TO THE OVUM IN ARTIFICIALLY INSEMINATED SUPER- AND SINGLE-OVULATING CATTLE?

R.G. Saacke,[1] J.M. DeJarnette, J.H. Bame, D.S. Karabinus and S.S. Whitman

Department of Dairy Science, Virginia Polytechnic Institute and State University
Blacksburg, VA 24061

Received for publication: August 25, 1997
Accepted: March 23, 1998

ABSTRACT

The collective efficiency of barriers in the female tract against spermatozoa with abnormal heads was studied. In Experiment 1, Day 6 ova/embryos were recovered nonsurgically from superovulated (n=24) and single-ovulating (n=44) cows following artificial insemination with semen of bulls selected for normal spermatozoal motility (≥50%) and high content (>30%) of spermatozoa with misshapen heads, random nuclear vacuoles or the diadem defect. To assess characteristics of spermatozoa capable of traversing barriers in the female tract, accessory spermatozoa were classified morphologically (x 1250) and compared with those of the inseminate. Superovulated cows proved inadequate for assessment of accessory spermatozoa due to evidence of poor sperm retention in the zona pellucida; thus, only single-ovulating cows were used. Accessory spermatozoa (n=479) from 31 ova/embryos recovered from 44 cows were more normal in head shape than those in the inseminate (76 vs 62%; $P<0.05$). Spermatozoa with normal head shape, but with nuclear vacuoles appeared as accessory spermatozoa at the same frequency as they were found in the inseminate (20 vs 17%, respectively). Only sperm cells with subtly misshapen heads appeared as accessory spermatozoa. In Experiment 2, semen pooled from 4 bulls having large numbers of spermatozoa exhibiting a gradation from severely asymmetrically misshapen heads to subtly misshapen heads was evaluated. Again, the accessory sperm population (960 sperm cells recovered from 64 ova/embryos) was enriched with spermatozoa of normal head shape relative to the inseminate (53 vs 26%, respectively; $P<0.05$). Sperm cells with only nuclear vacuoles and those with subtly misshapen heads were not different between the accessory and inseminate populations (11 vs 8%, and 20 vs 25%, respectively). We conclude that morphologically abnormal spermatozoa are excluded from the accessory sperm population based upon severity of head shape distortion.

Key words: abnormal spermatozoa, sperm transport, accessory spermatozoa

Acknowledgments

This research was supported by Select Sires Inc., Plain City, OH, and the National Association of Animal Breeders, Columbia, MO.

[1] Correspondence and reprint requests.

Theriogenology 50:117–128, 1998

0093-691X/98/$19.00
PII S0093-691X(98)00119-8

INTRODUCTION

The proportion of spermatozoa with abnormal morphology in semen has been associated with subfertility and sterility in the bovine (23, for review). Differences among types of spermatozoal abnormalities, with respect to their impact on fertility, were recognized by early workers (10,26,27). Such differences may be based upon how a specific abnormality disrupts reproduction. Bulls differ in the numbers of spermatozoa required for maximum fertility (24) and in the maximum fertility, which is ultimately achievable, regardless of sperm dosage (5). On this basis reproductive failure due to semen deficiencies may be considered as either compensable (i.e., capable of being reduced or overcome by increased sperm dosage to the female) or uncompensable (i.e., where sub-fertility due to the male is unresponsive to increased sperm dosage). A possible scenario for this concept would be the existence of spermatozoa with deficiencies that are removed at specific barriers in the female tract, thus prohibiting such sperm cells from competing with normal spermatozoa for fertilization. Immotile spermatozoa (18) or those with tail defects or protoplasmic droplets (which interfere with motility) are markedly impaired at barriers in the female reproductive tract (15). Such a deficiency would be compensable if a sufficient number of normal live spermatozoa were present to optimize contact with the ovum and sustain the fertilization process. On the other hand, abnormal spermatozoa capable of traversing barriers and competing for fertilization would impact fertility in proportion to their level in the inseminate if such spermatozoa could preempt a normal spermatozoon by initiating fertilization and (or) embryogenesis but not sustain either event. Such a deficiency would be uncompensable because fertility of the male would be, expectedly, unresponsive to sperm dose. These uncompensable sperm characteristics may be the more serious defects of semen impacting fertility referred to by the earlier investigators cited above (10,26,27). Accessory spermatozoa (spermatozoa entrapped in the zona pellucida by the "zona reaction" following penetration of the fertilizing sperm cell) have provided a basis for quantifying the number and quality of spermatozoa competing for fertilization in cattle. Quantitatively, the accessory sperm number has been positively associated with fertility (28) and embryo quality (4,16). The morphology of accessory spermatozoa in relation to those in the inseminate has received little attention in cattle. However, accessory spermatozoa would have had to traverse the barriers in the bovine reproductive tract as well as undergo capacitation, ovum recognition and binding, the true acrosome reaction and partial penetration of the zona pellucida. Thus, accessory spermatozoa appear to represent our best opportunity for evaluating spermatozoa that would exhibit uncompensable semen traits.

The objective of this study was to compare the morphological characteristics of accessory spermatozoa with those of the inseminate, with the possibility that potentially uncompensable spermatozoa could be identified. Specifically, bovine spermatozoa with classifiably abnormal heads were evaluated with respect to their ability to qualify as accessory spermatozoa following artificial insemination in the uterus.

MATERIALS AND METHODS

Experiment 1

A single ejaculate from each of 4 bulls was chosen based upon semen content of spermatozoa having relatively normal viability (>50% motile, >60% intact acrosomes) and high proportions of abnormal sperm head morphology (>30%). Each ejaculate was extended in egg-yolk-citrate-glycerol and cryopreserved at 100 x 10^6 sperm/ml in 0.5-mL French straws (IMV, l'Aigle, France) according to conventional procedures (19), which included thawing straws in a water bath at 37 °C for 30 sec prior to use. Naturally cycling, single-ovulating cows (n=44) or cows conventionally superovulated (n=24) using 50 mg of FSH-P (Shering-Plough, Kenilworth, NJ) over 4 d and 40 mg $PGF_{2\alpha}$ (Lutylase, Pharmacia and Upjohn Co., Kalamazoo, MI) on Day 4 were artificially inseminated in the uterine body with 1 mL of semen (100 x 10^6 cells) from the pooled contents of 5 straws from 1 of the 4 ejaculates. Cows were observed for estrus twice daily (early morning and early evening), and were inseminated once approximately 12 h after first observation of standing for mounting by a herdmate. A second insemination was made for superovulated cows if they were still standing 12 h after the first insemination; however, less than 10% of the cows were inseminated twice. Each ejaculate was differentially counted for abnormal sperm cells using Nomarski differential interference contrast optics (DIC) at x1250 magnification. The counts were conducted on wet smears after arresting sperm motility by the addition of 40 mM sodium fluoride (1:1, v/v; 14). Two differential counts of 100 cells each were averaged to characterize each ejaculate. Misshapen heads were given first priority in classification, and nuclear vacuoles, distributed in random locations (craters) or uniformly at the nuclear ring (diadem defect), were classified with second priority. While nuclear vacuoles could have appeared in both misshapen heads and normally shaped heads, the classification of craters (3) or diadem sperm (1) was reserved for spermatozoa containing the vacuoles, but in a normally shaped head. In addition to the initial classification, the thawed extended semen of each ejaculate was incubated at 37°C and examined again 12 h post thawing to determine if there was differential aging or death of abnormal compared with normal sperm cells. Duration of viable sperm life was based upon the integrity of the acrosome, as indicated by the presence of the apical ridge (21).

Six days after insemination, ova/embryos were recovered nonsurgically using standard uterine flushing techniques. To reveal and evaluate accessory spermatozoa, the procedure of DeJarnette et al. (4) was employed. This technique involved partial digestion of the zona pellucida with a protease (Pronase, Behring Diagnostics, La Jolla, CA) in a hanging-drop preparation, followed by compression of the ovum/embryo with a coverslip and examination of the unfixed smear using DIC optics at x1250 magnification. Using this procedure, nearly all heads of accessory spermatozoa were rendered flat so their morphology could be critically evaluated. Morphological classification of the accessory spermatozoa

was the same as that described for the inseminate above, with the exception that occasional sperm cells were omitted if they did not present a flat aspect to the viewer, thus precluding accurate classification.

Experiment 2

Based upon results of Experiment 1, we deemed it advisable to investigate semen where there was a distinct gradient in severity of misshapen sperm heads. Ejaculates accepted for use were based upon the same criteria as for Experiment 1; however, semen for this experiment was obtained by pooling qualifying ejaculates of 4 bulls. The pooled semen was cryopreserved in the same manner as described in Experiment 1; however, the insemination dose was 40 x 10^6 cells/straw. Of interest in the semen of these bulls were the asymmetrically misshapen sperm heads which were sufficiently abundant and varied from severe to subtle. Furthermore, only single-ovulating cows were inseminated for reasons presented in the Results of Experiment 1.

Statistical Analysis

For Experiment 1, the mean percentage of normal and abnormal spermatozoa for the inseminate were calculated from the 4 possible inseminates. Data were pooled across these 4 ejaculates for presentation and statistical analysis by calculating weighted means, where weighting was based on the proportion of the total number of accessory spermatozoa attributed to a given ejaculate. That is, if one-third of the accessory sperm cells were attributable to Ejaculate 1, data for that ejaculate would be weighted to represent 33.3% of the reported mean for the inseminate. For Experiment 2, all oocytes/embryos (n=64) were derived from the same inseminate, which was produced by pooling the ejaculates of 4 bulls. Comparisons of accessory sperm parameters for single vs superovulated cows were made using the Student's *t*-test (Experiment 1). For comparison of spermatozoal morphological traits of inseminated vs accessory sperm populations, Chi-square analysis was carried out in a 6 x 2 (5 df) and 7 x 2 (6 df) contingency table for Experiments 1 and 2, respectively. A Bonferroni adjustment was used to maintain α levels in cases of multiple use of the same data in the Chi-square analyses, where frequencies of specific abnormalities were compared.

RESULTS

Experiment 1

Quantitative fertilization and accessory sperm data for superovulated and single-ovulating cows inseminated with the same semen are presented in Table 1.

Table 1. Comparison of superovulated versus single-ovulating cows with respect to fertility and accessory sperm following AI with frozen semen characterized by high content of abnormal sperm heads (>30%) and normal viability (>50% motile) (Experiment 1)

Characteristic	Superovulated	Single-ovulating
Number of cows	24	44
Number of ova/embryos recovered	155	31
% ova fertilized	64.5	83.8
% fertilized ova with accessory sperm cells	10	61*
Median (range) number of accessory spermatozoa per ovum/embryo recovered	0 (0 - 15)	2.5 (0 - 187)
Number of accessory sperm cells per ovum/embryo having accessory spermatozoa (mean ± SD)	1.1 ± 2.1	21.1 ± 30.5
Number of accessory sperm cells per cow having accessory spermatozoa (mean)	0.7	9.0*

*$P<0.05$; other values not tested for significance.

Superovulated cows had fewer fertilized ova possessing accessory sperm cells than single-ovulating cows (10 vs 61%; $P<0.05$; Table 1), and the number of accessory sperm cells per cow was higher for single-ovulating cows than for superovulated cows (9.0 vs 0.7 sperm/cow, respectively; $P<0.05$; Table 1) despite an average ovum recovery of 6.5 vs 1.0 for super- and single-ovulating cows, respectively. Based upon the mean ± SD and median accessory spermatozoa per ovum/embryo, it is clear that accessory spermatozoa are not distributed normally. It also appears that many more spermatozoa are available to the single-ovulating ovum compared with the superovulated ovum when the mean number of accessory sperm cells per ovum/embryo with accessory sperm cells is examined (1.1 vs 21.1 sperm cells per ovum/embryo; Table 1). However, upon close examination of the ovum/embryo smears using DIC optics at x1250, there was evidence of sperm tracks in approximately 40% of the zonae from superovulated cows, indicating that accessory spermatozoa may have been lost between the time of fertilization and ovum/embryo recovery. Only on rare occasion was a sperm track found in single-ovulating ova/embryos, with the exception of a single track in a fertilized ovum having no accessory sperm cells (presumably the track of the fertilizing sperm). Since sperm tracks were not quantified in this study, rendering accessory sperm comparisons among single and superovulating cows of little biological importance, statistical treatment of the accessory spermatozoa per ovum/embryo between single and superovulated cattle was not applied. On the basis that accessory sperm number is quite low in superovulated ova and that accessory spermatozoa from superovulated ova may not accurately reflect the population of sperm competing for fertilization, the superovulated cows were excluded from the accessory sperm data reported in this experiment.

From 31 single-ovulating cows in this experiment, 479 accessory spermatozoa were recovered and classified for type of head abnormality. A comparison of percentage of head abnormalities between those calculated for the inseminate (weighted from the 4 ejaculates) and the accessory spermatozoa derived from the weighted inseminate is presented in Figure 1. Inseminated sperm characteristics differed from those of accessory spermatozoa ($P<0.05$, $\chi^2=54.2$ with 5 df).

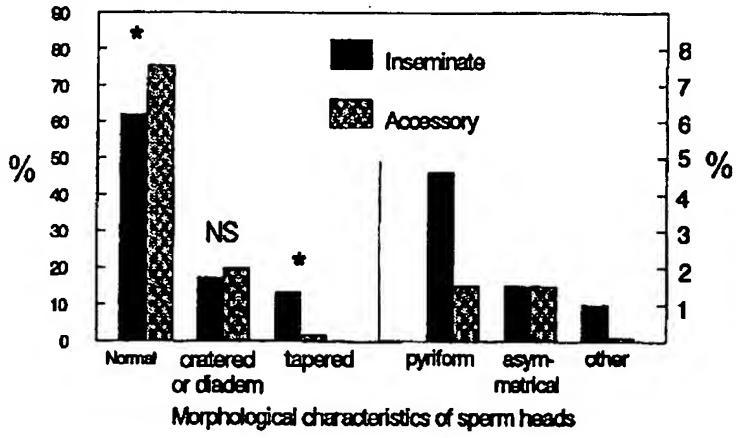

Figure 1. Morphological characteristics of accessory sperm heads compared with those in the inseminate (Experiment 1). Higher frequency head characteristics are presented on the left scale and lower frequency characteristics on the right scale. Only the percentage of normal spermatozoa, cratered/diadem defects and tapered heads were tested statistically using Bonferroni adjustments of Chi-square analysis. NS = nonsignificant, *Significant ($P<0.05$).

There was a significant enrichment of sperm with normal heads from the inseminate to the accessory sperm population (62 vs 76%, respectively). The highest single defect in the inseminate were sperm cells with normal head shape but having nuclear vacuoles in the form of randomly distributed vacuoles termed "craters" (3) and the diadem defect (1), defined as a string of nuclear vacuoles (2 or more) arrayed in a line along the nuclear ring. Cratered or diadem spermatozoa did not differ between the inseminate and accessory sperm populations (17 vs 20%, respectively). Although the craters and diadem defect were evident in sperm cells with misshapen heads, classification priority was given to type of misshapen head. In this respect, the significant improvement in normal accessory spermatozoa over that of the inseminate was due to the collective discrimination against tapered, pyriform and "other" head shape abnormalities (short, giant, micro). Due to the low frequency of specific abnormalities, statistical testing of

each for differences between the accessory and inseminate populations was not conducted. Accessory spermatozoa classified as either tapered or pyriform were quite subtle in their deformity, suggesting that severity of head shape may dictate the accessibility of spermatozoa to the ovum. Based on 12-h in vitro incubation at 37°C of cryopreserved spermatozoa of the inseminate, differential death of spermatozoa with normal vs abnormal heads as judged by acrosomal integrity did not occur (data not presented).

Experiment 2

In this experiment, a total of 64 ova/embryos was collected, yielding 960 morphologically classifiable accessory sperm cells. Accessory sperm head morphology differed from that in the inseminate ($P<0.05$, $\chi^2=112.9$ with 6 df). The morphological comparison of inseminate and accessory sperm is presented in Figure 2. Spermatozoa with normal heads were again greater in the accessory sperm population than in the inseminate (53 vs 26%, respectively; $P<0.05$). Sperm cells with craters in otherwise normally shaped heads (classified as cratered) were not different between accessory spermatozoa and those of the inseminate (11 vs 8%, respectively). Accessory sperm categories of tapered, long and asymmetrical were lower than those in the inseminate ($P<0.05$). Due to the low frequency of pyriform sperm cells, this category was not tested statistically. Slightly asymmetrical heads did not differ between inseminate and accessory sperm populations (25 vs 20%, respectively).

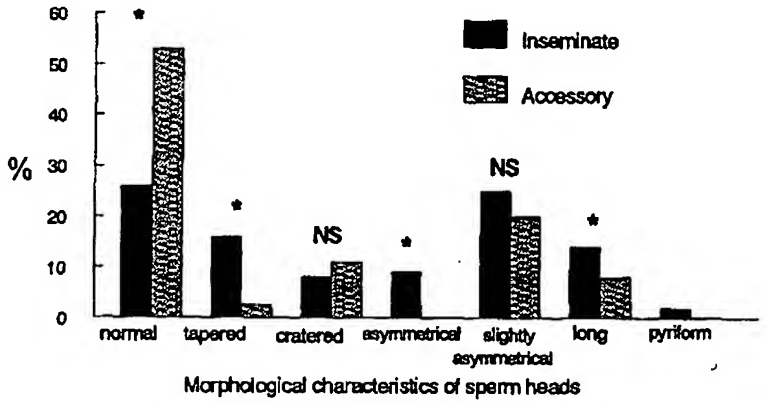

Figure 2. Morphological characteristics of accessory sperm heads were compared with those of the inseminate (Experiment 2). All categories except pyriform were tested statistically using Bonferroni adjustments of Chi-square analysis. NS = nonsignificant and *Significant at $P<0.05$.

DISCUSSION

In Experiment 1, it was clear that the number of accessory spermatozoa that were available for analysis were markedly reduced in superovulated cows compared with single-ovulating cows (0.7 vs 9.0 accessory sperm cells per cow for superovulated and single-ovulated cows, respectively; Table 1), making the superovulated cow quite inefficient for the type of study we undertook. The data might be interpreted to indicate that sperm transport was impaired in the superovulated cow based upon the lower total number of accessory sperm cells per cow, despite the difference in number of ova released (6.5 vs 1.0 for the super- and single-ovulating cow, respectively). Although this may be true, sperm tracks could be observed in the zonae pellucidae of approximately 40% of the fertilized ova of superovulated cows, while only on rare occasion were tracks observed in the zonae of single-ovulating fertilized ova. The tracks were most apparent when focusing through the zona rather than on any single plane within the zona. This is due to the shallow depth of focus achieved with DIC optics at magnifications of x400 and higher. Thus, it appears that accessory sperm loss from the zona pellucida may have occurred between the time of fertilization and ovum/embryo recovery (6 d following insemination) in superovulated cows. This is also indicated by the considerably higher proportions of superovulated ova having accessory spermatozoa (18%), as reported by Hawk and Tanabe (8) recovering tubal ova/embryos at Day 3 post insemination compared with the 10% rate in our study, in which uterine embryos were recovered 6 d post insemination. Nevertheless, in our study such a loss of spermatozoa in superovulated cows would make questionable the interpretation of sperm numbers available for fertilization as well as the quality of spermatozoa available for analysis for superovulated cattle.

An explanation for the accessory sperm loss from superovulated ova/embryos may reside in a difference in the nature of the zona pellucida itself between superovulated and single-ovulating cattle. The time required for protease digestion of the zonae pellucidae of ova/embryos recovered 6 d after insemination has been observed to be approximately twice as long for single-ovulating cows as for superovulated cows (22). This has been interpreted to reflect a potential difference in composition or hardness of the zona pellucida between these cows, and has interesting implications, particularly in view of the findings of Howard et al. (9), who indicated the sperm selection potential of the zona pellucida favoring spermatozoa with normally-shaped heads. In cats, the above workers showed that as layers of zona pellucida were sampled from the outside in, accessory spermatozoa trapped therein became more normal in shape. Differences among cows or hormonal regimens associated with ovum maturation and ovulation affecting zona composition or hardness could readily impact the pregnancy rate by altering the selectivity of this potentially important barrier and (or) the sperm transport system in general. It has also been shown, using quantitative accessory sperm data, that insemination of very high sperm numbers may be required to optimize sperm accessibility to the ovum in superovulated cattle (7). On the basis

of this discussion and the objectives of our study, we deemed it important to limit our observations on the morphology of accessory spermatozoa to only single-ovulating cows.

In both Experiments 1 and 2 there was a distinct increase in normal cells in the accessory sperm population compared with that in the inseminate (Figures 1 and 2). In Experiment 1, the weighted inseminate contained appreciable levels of abnormal sperm heads (38%), more than half of which (21%) were abnormal due to a variety of shapes (tapered, pyriform, asymmetrical etc.), the other portion (17%) due to nuclear content of randomly distributed nuclear vacuoles or the diadem defect, but in otherwise normally shaped heads. There were also vacuoles in the abnormally shaped heads; however, classification was first made by shape, then by nuclear vacuoles, the only other head defect found. Since normal heads were increased in the accessory sperm population and the head defects associated only with vacuoles were not different between accessory and inseminate populations, head shape appeared to be the discriminating factor. In addition, misshapen heads were subtle and, therefore, led to Experiment 2, in which there were distinct populations of asymmetrical heads ranging in severity of distortion. Based upon the findings in Experiment 2 that subtle distortions of the head are found to be nearly equally distributed in accessory and inseminated sperm populations, we find that we are in agreement with the work of Krzanowska et al. (11,12) in the mouse, showing that discrimination of spermatozoa competing for fertilization is dependent on the severity of head shape distortion. Recognizing the importance of sperm motility in accessibility to the ovum in vivo, as clearly shown in the rabbit (18), the suggestion has been offered that small geometrical differences in head morphology could cause large differences in sperm hydrodynamics (6). Thus, impaired or aberrant sperm motility could be the underlying basis for sperm exclusion due to head morphology. It has been reported recently that severely misshapen sperm heads are more frequently found in the dead than live cell population of an ejaculate (17). However, marked changes in sperm head morphology without a change in sperm motility has also been reported to occur in response to mild increases in scrotal temperature (25). In Experiment 1, a difference in viable life of normal and abnormal spermatozoa was not observed when acrosomal integrity was used as a measure of viability.

From a practical standpoint and of importance to semen evaluation for artificial insemination, it appears that abnormal head morphology can have both a compensable and an uncompensable component. Spermatozoa with severely misshapen heads undoubtedly do not compete for fertilization due to, as yet, unknown barriers or levels of viability. Therefore, additional spermatozoa in the insemination dose would probably be needed to overcome a deficiency in meeting the female threshold requirements for sperm dose, as previously postulated by Sullivan and Elliott (24). However, it appears that spermatozoa with subtle deviations in shape or with normal shape but nuclear vacuoles can access the ovum, and probably do compete for fertilization. Indirect evidence has been presented earlier (4,13) that semen high in abnormal sperm head counts results in

Increased proportions of degenerated and low-quality embryos, indicating the presence of incompetent spermatozoa or the uncompensable factor. Since spermatogenic disturbance in males usually results in pleomorphic spermatozoa varying in severity of shape (2,20), the impact on pregnancy rate may be due to both compensable and uncompensable factors. The impact of specific sperm deformities on fertilization and embryo quality would appear to be the next step in characterizing the importance of sperm morphology to the pregnancy rate in cattle and the importance of semen evaluation. The application of new molecular methods addressing the quality of DNA and its associated proteins in morphologically normal and subtly abnormal spermatozoa in ejaculates characterized as containing above average quantities of abnormal spermatozoa also needs to be considered. It would seem plausible that perturbations of spermatogenesis signified by the presence of abnormal spermatozoa may extend to normal appearing cells in such samples, which we have yet to recognize through current tests.

REFERENCES

1. Bane A, Nicander L. Pouch formation by invaginations of the nuclear envelope of bovine and porcine sperm as a sign of disturbed spermatogenesis. Nord Vet Med 1965;17:628-632.
2. Barth AD, Oko RJ. Abnormal Morphology of Bovine Spermatozoa. Ames, IA: State University Press, 1984;130-192.
3. Coulter GH, Oko RJ, Costerton JW. Ultrastructure of "crater" defect of bovine spermatozoa. Theriogenology 1978;9:165-168.
4. DeJarnette JM, Saacke RG, Bame J, Vogler CJ. Accessory sperm: their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J Anim Sci 1992;70:484-491.
5. den Daas N. Laboratory assessment of semen characteristics. Anim Reprod Sci 1992;28:87-94.
6. Dresdner RD, Katz DF. Relationship of mammalian sperm motility and morphology to hydrodynamic aspects of cell function. Biol Reprod 1981;25:920-930.
7. Hawk HW, Conley HH, Wall RJ, Whitaker RO. Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. Theriogenology 1988;29:1131-1138.
8. Hawk HW, Tanabe TY. Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. J Anim Sci 1986;63:551-560.
9. Howard JG, Donoghue AM, Johnston LA, Wildt DE. Zona pellucida filtration of structurally abnormal spermatozoa and reduced fertilization in teratospermic cats. Biol Reprod 1993;49:131-139.

10. Lagerlof N. Morphological studies on the changes in the sperm structure and in the testes of bulls with decreased or abolished fertility. Acta Path Microbiol Scand 1934;19:254-267.
11. Krzanowska H. The passage of abnormal spermatozoa through the uterotubal junction of the mouse. J Reprod Fertil 1974;38:81-90.
12. Krzanowska H, Lorenc E. Influence of egg vestments on in vitro penetration of mouse eggs by misshapen spermatozoa. J Reprod Fertil 1983;68:57-62.
13. Miller D, Hurdka M, Cates WF, Mapletoft R. Infertility in a bull with a nuclear sperm defect: a case report. Theriogenology 1982;17:611-621.
14. Mitchell JR, Hanson RD, Fleming WN. Utilizing differential interference contrast microscopy for evaluating abnormal spermatozoa. Proc 7th Nat'l Assoc Anim Breeders Conf Artif Insem Reprod 1978;64-68.
15. Mitchell JR, Senger PL, Rosenberger, JL. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. J Anim Sci 1985;61:956-967.
16. Nadir S, Saacke RG, Bame J, Mullins J, Degelos S. Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. J Anim Sci 1993;71:199-204.
17. Ostermeier GC, Parrish JJ. Objective assessment of the nuclear shape differences which exist between live and dead bovine sperm cell populations. Biol Reprod 1997;56(Suppl 1):212 abstr.
18. Overstreet JW, Cooper W, Katz DF. Sperm transport in the reproductive tract of the female rabbit. II. The sustained phase of transport. Biol Reprod 1977;19:115-132.
19. Robbins RK, Saacke RG, Chandler PT. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. J Anim Sci 1976;42:145-154.
20. Saacke RG. What is abnormal and is abnormal dependent upon the animal (a discussion on sperm morphology). Proc 13th Nat'l Assoc Anim Breeders Conf Artif Insem Reprod 1990;67-73.
21. Saacke RG, Marshall CE. Observations on the acrosomal cap of fixed and unfixed spermatozoa. J Reprod Fertil 1968;511-515.
22. Saacke RG, Nadir S, Dalton J, Bame J, DeJarnette JM, Degelos S, and Nebel RL. Accessory sperm evaluation and bull fertility. Proc 15th Nat'l Assoc Anim Breeders Conf Artif Insemin Reprod 1994;57-67.
23. Sullivan JJ. Morphology and motility of spermatozoa. In: Salisbury GW, VanDemark NL, Lodge JR (eds), Physiology of Reproduction and Artificial Insemination of Cattle. San Francisco: WH Freeman, 1978;286-328.
24. Sullivan JJ, Elliott FI. Bull fertility as affected by an interaction between motile spermatozoa concentration and fertility level in artificial insemination. VI Int'l Cong Anim Reprod Artif Insem 1968;2:1307 abstr.
25. Vogler CJ, Bame JH, DeJarnette JM, McGilliard ML, Saacke RG. Effects of elevated testicular temperature on morphology characteristics of ejaculated spermatozoa in the bovine. Theriogenology 1993;40:1207-1219.

26. Williams WW, Savage A. Observations on the seminal micropathology of bulls. Cornell Vet 1925;15:353-375.
27. Williams WW, Savage A. Methods of determining the reproductive health and fertility of bulls. A review with additional notes. Cornell Vet 1927;374-376.
28. Wilmut I, Hunter RHF. Sperm transport into the oviducts of heifers mated early in estrus. Reprod Nutr Dev 1984;24:461-465.